A Direct Binding Scintillation Proximity Assay for Measuring Enzyme Activity

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Date:

We have proposed the use of direct absorption for SPA (scintillation proximity assay) before (see Disclosure by Zhengyu Yuan and Dawn (Zhong-Xiao) Chen of . SPA has been widely used for assays of protein-protein and protein-ligand interactions. But they all involve specific protein recognition interaction. To this date, all applications reported for enzymatic assay involve receptors for the reaction product or the derivative of the product.

While coating scintillant-containing solid phase with protein receptor to capture radio isotope labeled ligand was very useful assay method, it is not applicable to those assays in which there is no antibody or receptor for both substrate or product. For these type of assays, one has to either generate a specific antibody or derivatize the reaction substrate of product with molecules which is the legend for existing antibody. The latter process often fails due to the incompatibility of the derivatized chemicals as substrate for enzyme to recognize.

We propose to use non-specific interaction as the binding force to selectively absorb certain chemicals to the scintillant-containing solid phase. This idea was based on that during certain enzyme catalyzed reaction, there is profound physical properties difference between substrate and reaction product, such as charge difference or hydrophobicity difference. By using SPA solid phase and isotope labeled chemicals, one can selectively monitor the consumption of reaction substrate or the generation of the product without involve any specific protein interaction.

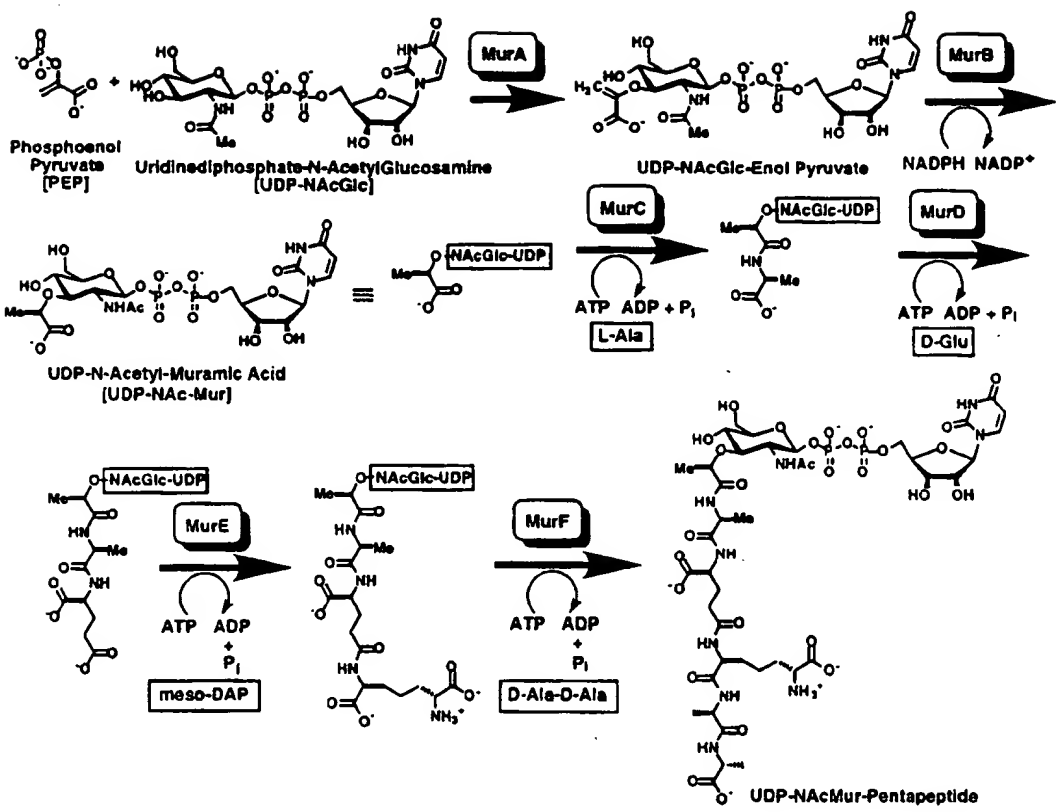
To be more specific, we propose the following:
1) Coat scintillant-containing surface with molecules with desired properties, such as positively (or negatively) charged molecules, or very hydrophobic surface;

2) Using isotope labeled substrate which has very different charge or hydrophobicity properties than the product. Such as use a neutral or positively charged substrate, but the reaction product will be negatively charged;
3) At the end of the reaction, simply transfer the reaction mixture to contact the specially coated SPA surface, such as a positively charged surface. For the isotope labeled substrate. it will not bind onto the positively charged surface but only the reaction product will. Therefore, only those isotope which is incorporated into the reaction product will be in close proximity to the scintillant and generate the signals, and by monitoring the signal, we can follow the reaction progress.

The followings are one of the examples:

Activity of many enzymes are difficult to measure due to the lack of easy detectable signal change such as color or fluorescence intensity changes. Quite often, it relies on chromatography method to separate substrate from reaction product and measure the separated chemicals. This process is time consuming and often not compatible to high throughput screening.

One of such examples is the sequential biosynthesis of Mur-pentapeptide. Starting with UDP-Glu-NAc and using ATP as energy source, six enzymes are used to add different amino acids consecutively onto the growing UDP-Glu chain to form the final pentapeptide product:



The reaction progress of each step in this cascade is difficult to measure due to the lack of chromaphore or flourophore change. The current assaying methods are either based on phosphate release, which is insensitive and can not distinguish alternative substrate inhibitor, or based on isotope labeling, which involves the separation of reaction substrate from product by chromatography method. In addition, one major difficulty in assaying these enzymes is the need to prepare the corresponding substrate for each step which is not commercially available and proven to be a very difficult process. As a matter of fact, that is the reason for not being able to run high throughput screening for these enzymes.

By using the method described above, we can prepare a positively charged surface on the FlashPlate. Although there are commercially available positively charged FlashPlate, it has quite low binding capacity. We have used methyltrioctylammonium bromide to coat the plate which could produce a positively charged surface with much higher binding capacity. Then, using tritium labeling the proper amino acid, and non-labeled other reaction substrate, together with enzymes, the tritium labeled amino acid will be incorporated into the UDP-containing product, which can easily bind to the positively charged surface under acidic condition while the parent tritiated amino acid will not bind to the same surface. For

example, with UDP-Glu-NAc, all the substrates in the pathway, and the tritiated D-Ala-D-Ala, together with all six enzymes (Mur A to F), at the end of the reaction, transfer the reaction mixture to the positively coated FlashPlate under acidic solution. Only those tritiated D-Ala-D-Ala which has been incorporated onto the UDP-containing chain will bind to the plate and generate signals while the intact D-Ala-D-Ala will not bind to the plate and has no effect. Therefore, the signal generated in this assay reflect the overall activity of 6 enzymes. Alternatively, by using tritiated L-Ala, the signal only reflects the overall activity of first three enzymes (Mur A to C) but independent of the subsequent enzyme reactions. Like wise, using different tritiated amino acid, one can easily follow the reaction steps of interest. In general, in order to detect enzymatic activity change of any steps in the pathway, one would adjust the enzyme/substrate concentration in such that concentration change of any enzyme will result in the decrease of the scintillation signal. However if any one particular reaction steps is of interest, one can increase the concentration of all other enzymes dramatically except the enzyme of interest, therefore, only the change of that enzyme concentration will result in scintillation signal changes.

There are two very important advantages of this assay:

1) It no longer needs to prepare the UDP-containing substrates for each individual enzymatic reaction steps, such as UDP-Mur-NAc, or UDP-Mur-NAc-amino acid(s). Start with commercially available UDP-Glu-NAc and amino acids, simply by adjusting the enzyme concentration and using the proper tritiated amino acid, one can follow the reaction progress of any steps of interest;

2) The detection method is extremely simple. It does not involve any separation. In addition, it does not need to prepare any antibody or receptors which are very difficult to prepare, nor it need to prepare special derivatized substrate which is both difficult and often incompatible to the enzymes.

It is also important to point out that these type of assay is particularly easy to be converted to high throughput format due to the ease of substrate supply and reaction signal detection which does not involve any separation steps.

To validate these concept, we have do the following experiments:

1) Mur A to C reaction:

In this experiment, reaction mixture containing:

Mur A,

Mur B,

Mur C

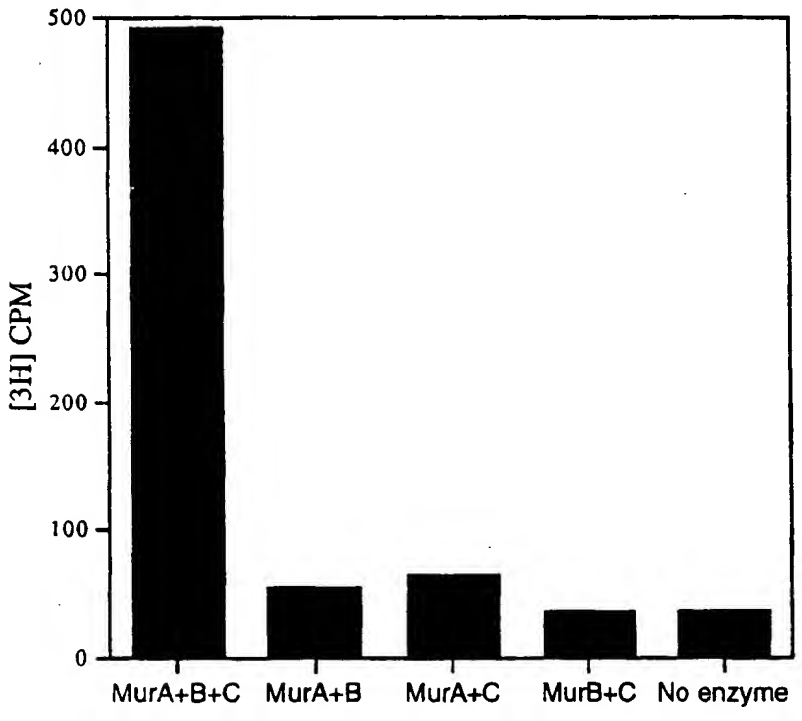
UDP-Glu-NAc

PEP
NDAPH
ATP
tritiated L-Alanine

MurA condenses UDP-Glu-NAc and PEP and its product is subsequently reduced by MurB using NADPH to produce UDP-Mur-NAc. MurC will add L-Ala to UDP-Mur-NAc while hydrolyze the ATP to ADP + Pi. At the end of the reaction, simply transfer the reaction mixture to positively coated FlashPlate which contain acetic acid solution and count the scintillation signal in the Top Counter. As indicated in the graph, when all the reaction substrate and enzymes were present, it generated a significant signal. If any one of these three enzymes were missing, only background signal can be detected, which validates the assay hypothesis.

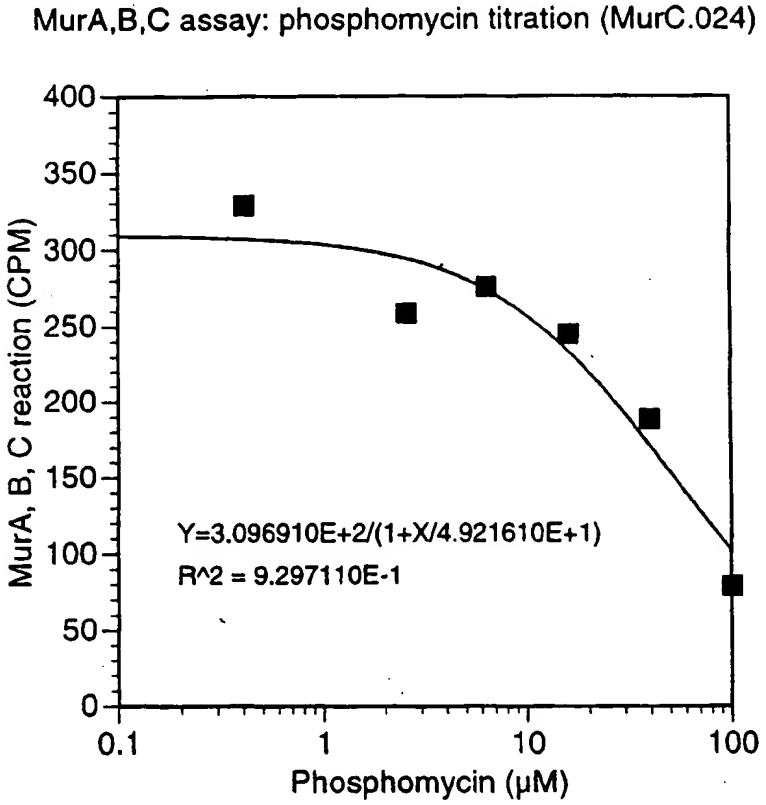
Figure 1:

MurA, B, C assay with flash plate (MurC.018)



2) Use Mur A to C one-pot reaction to detect inhibitor of MurA enzyme:
In this experiment, we used the assay system in Figure 1, and included different concentration of phosphomycin which is a know inhibitor of MurA. In this experiment, the Phosphomycin was not preincubated with UDP-Glu-NAc prior adding the rest of reaction substrate. This experiment shows that this assay is able to detect inhibitors of individual enzyme in the pathway.

Figure 2:



3) Using the direct binding solid phase scintillation assay to assay Mur A to F in one reaction mixture:

The reaction mixture contains:

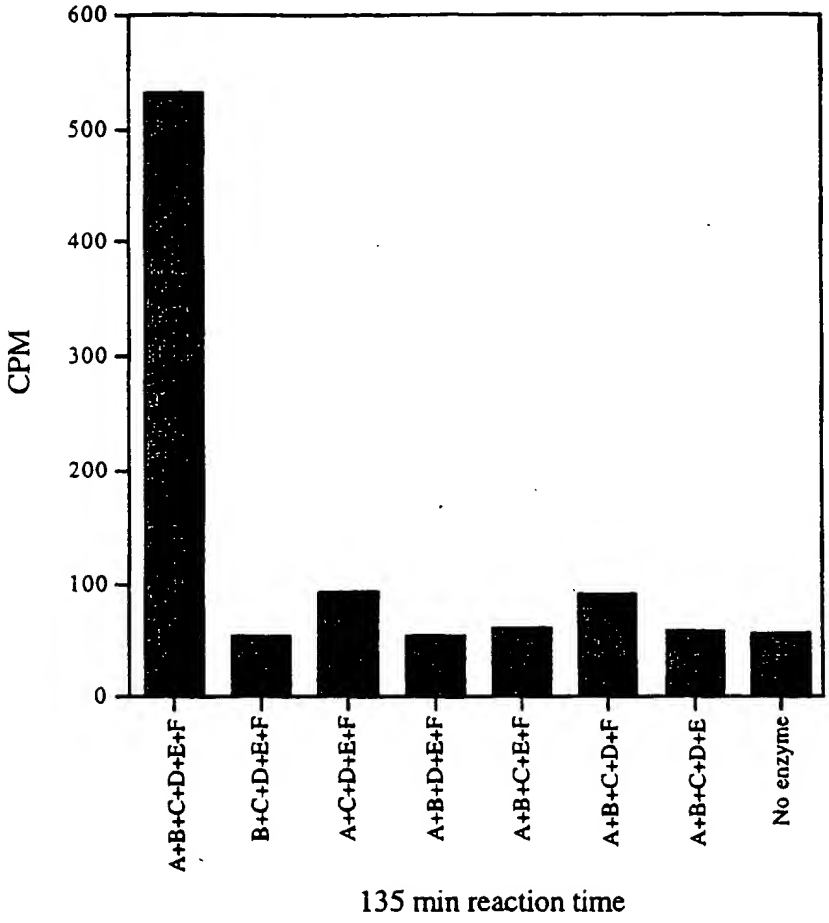
Mur A
Mur B
Mur C
Mur D
Mur E
Mur F
UDP-Glu-NAc
PEP
NDAPH
ATP
L-Alanine
D-Glutamate
D,L-diaminopimelic acid
tritiated D-Ala-D-Ala

MurA condenses UDP-Glu-NAc and PEP and its product is subsequently reduced by MurB using NADPH to produce UDP-Mur-NAc. MurC, D, E, and F will add L-Ala, D-Glu, DAP, and D-Ala-D-Ala consecutively to UDP-Mur-NAc while hydrolyze the ATP to ADP + Pi. As indicated in the graph, when all the reaction substrate and enzymes were present, it generated a significant signal. If any one of these three enzymes were missing, only background signal can be detected, which validates the assay hypothesis.

It is important to point out that the same reaction mixture was monitoring the activity of six different enzymes, and use only commercially available substrates.

Figure 3:

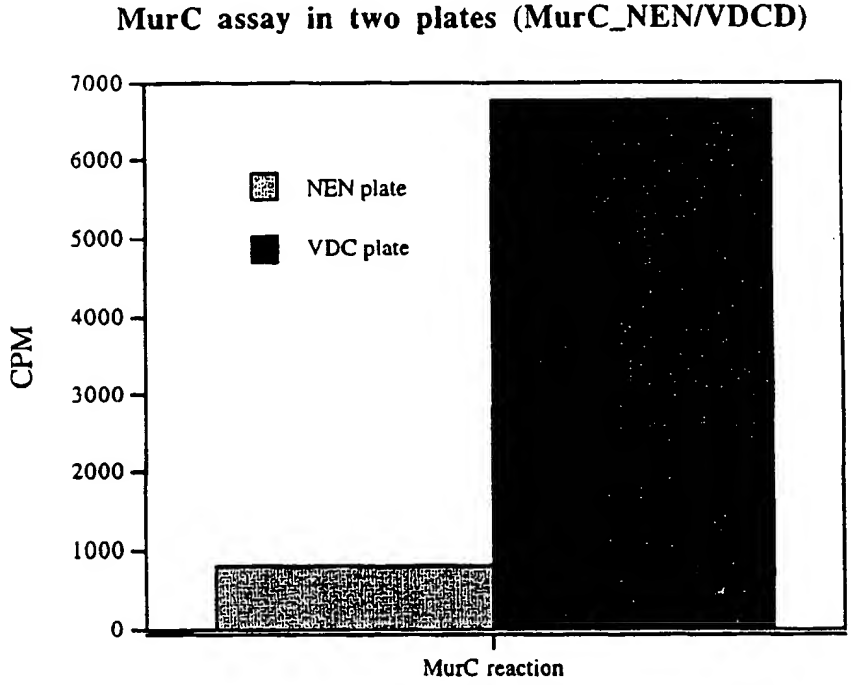
MurA to F six-in-one pot assay (MurC.046,)



4) Comparison of commercial FlashPlate and Versicor plate:

For the same reaction mixture from MurC reaction, it was suspended into two types of FlashPlate wells. One was comical plate which was coated with poly-D-lysine, the other was coated with methyltrioctylammonium bromide. The following Figure 4 clearly shows that Versicor's plate was significantly better than the comical plate in terms of binding capacity.

Figure 4:



This methodology can be easily apply to many other enzyme reactions:

1) Any one of the substrate is not negatively charged but the product which contains part of that substrate is negatively charged;

2) Any one of the substrate is not positively charged but the product which contains part of that substrate is positively charged;

3) Any one of the substrate is not very hydrophobic but the product which contains part of that substrate is very hydrophobic;

4) Any one of the substrate is negatively charged but the product which contains part of that substrate is not negatively charged;

5) Any one of the substrate is positively charged but the product which contains part of that substrate is not positively charged;
6) Any one of the substrate is very hydrophobic but the product which contains part of that substrate is not very hydrophobic;

For example, the kinase reaction:
Kinase adds a phosphate group onto peptide. We can prepare the tritiated kinase peptide substrate. The kinase will add phosphate group onto the peptide resulting a reaction product which is negatively charged under certain pH. That phosphorylated peptide will bind to the positively coated FlashPlate or any scintillant containing surface and produce scintillation signal, while the unphosphorylated peptide will not bind and remain undetectable.

For example, the lipase reaction:
Lipase removes fatty acid from lipid. We can prepare lipid with tritiated fatty acid chain. Under acidic pH, the fatty acid will be very hydrophobic while the lipid will remain in the solution. By coating the scintillant containing surface with very hydrophobic material, only the released fatty acid will bind to the solid surface produce scintillation signal.

For example, phosphatase:
Phosphatase remove phosphate group from a phosphorylated peptide. We can prepare the tritiated phosphorylated peptide. Once the phosphate group was removed from the parent peptide, it result a peptide which is not negatively charged and will not bind to positively charged surface. So in this case, we will monitor the reaction progress by following the scintillation signal decrease.

For example, proteases:
Protease hydrolysis peptide into two segment A and B. If A segment of the peptide contains either phosphate, or arginine but not B segment, we can tritiate the B segment of the original peptide. After the enzymatic reaction, the B segment along will no longer bind to the scintillant containing surface and reduce the scintillation signal.

For example, tRNA transferase:
tRNA transferase transfer an amino acid onto the RNA and ready for the protein synthesis. We can tritiate the amino acid which should not bind to the positively charged surface in the acidic solution. Once transfer to RNA, the whole amino acid-tRNA will bind to positively charged surface and generate scintillation signal.